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Cultivation of immobilized *Dictyostelium discoideum* for the production of soluble human Fas ligand

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Abstract *Dictyostelium discoideum* was immobilized by cultivation on inorganic porous matrices consisting of broken pumice or a ceramic catalyst carrier (CeramTec) to produce human soluble Fas ligand (hFasL). These supports were actively colonized by *D. discoideum* reaching cell (number) densities 10–20 times higher locally than those observed in suspension culture under similar conditions. In repeated batch or continuous operation, hFasL productivities of up to 15–25 μg h^{-1} l^{-1} pore volume were attained. The immobilized cell densities and hFasL productivities could be kept constant for a long period of time by repeated renewal or continuous feeding of complex or synthetic medium.

Introduction

Progress in genetic engineering allows high value therapeutics, vaccines, and recombinant proteins in general to be produced in bacteria, yeast, filamentous fungi, and baculovirus, as well as in plant and animal cells. The growing need for proteins as therapeutic agents can be met only by heterologous syntheses of recombinant proteins (Rai and Padh 2001). Many of these require post-translational modifications to be properly folded, active and stable. Therefore, new emphasis has been placed on development of eukaryotic expression systems (Jung and Williams 1997). For successful production of a protein in substantial quantities, integration of knowledge from genetic engineering, physiology, protein chemistry and biochemical engineering is required (Georgiou 1996; Neubauer and Winter 2001). Protein production is often strongly correlated with cell density. Thus, the first aim in process development is directed towards reaching high cell densities.

Immobilization is one efficient method of increasing cell density, and it even allows reactors to be operated continuously at high space velocity without wash-out of cells. This contributes to easy cell separation from products in solution, protection of cells from shear force, stable microenvironmental conditions (Tiltscher and Storr 1993), and the capability of long term continuous cultivation. Among the different immobilization procedures one of the mildest and most extensively used is adsorption of cells onto macroporous matrices (Fitch et al. 1998; Kennedy and Cabral 1983). *Dictyostelium discoideum* may be immobilized by inclusion into inorganic supports (Tiltscher and Storr 1993). However, it has been shown recently that *D. discoideum* actively colonizes porous carrier particles (Beshay et al. 2003a, b).

In general, the lower eukaryote and social amoeba *D. discoideum* is a promising host for the production of heterologous proteins requiring post-translational modifications (Glenn and Williams 1988; Emslie et al. 1995). In particular, it performs *N*-linked and *O*-linked glycosylation (Jung and Williams 1997) and can be cultivated on bacteria owing to phagocytosis or, with mutant strains, on soluble (axenic) growth media owing to pinocytosis.

In this study, immobilized *D. discoideum* is used to produce human soluble Fas ligand (hFasL), a protein that induces programmed cell death, or apoptosis, which consequently represents an interesting target for therapeutic intervention strategies in various chronic diseases (Borgerson et al. 1999; Rathmel et al. 1995; Russell et al. 1993; Tang et al. 1998). The characteristics of the strain used were reported recently, together with strategies to improve the productivity of hFasL in suspension culture (Lu et al. 2004). Immobilization was carried out by

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colonization of cheap inorganic porous supports like broken pumice and CeramTec, a ceramic catalyst carrier. In the present study, the cultivation of immobilized *D. discoideum* was investigated in repeated batch, as well as continuous, cultivations in the presence of either the axenic complex medium HL-5C or a modified synthetic FM medium (Franke and Kessin 1977), called SIH medium (Han et al. 2004a,b). These production strategies were analyzed with respect to cell density, product concentration and productivity.

Materials and methods

Chemical substances and media components

Yeast extract was purchased from Bioferm (Waldmünchen, Germany). Bactotryptone was supplied by Difco (Detroit, Mich.). Casein peptone and protease peptone were obtained from Merck (Darmstadt, Germany), as was D-glucose. Most amino acids were from Ajinomoto (Hamburg, Germany), only asparagine was obtained from ICN (MP Biomedicals, Eschwege, Germany), histidine from Senn Chemicals (Dielsdorf, Switzerland), and glycine from Carl Roth (Karlsruhe, Germany). Folic acid and dihydrostreptomycin sulfate were supplied by Sigma (St. Louis, Mo.), whereas geneticin (G-418) was from Serva (Heidelberg, Germany). Cyanocobalamin, lipoic acid, riboflavin, thiamine-HCl, biotin were purchased from Merck. All other chemicals were at least of analytical grade.

Strain used

D. discoideum AX3-strain, AX3-pCESFL95-G2 (AX3-95G2), having been transformed with pCESFL95, an MB12NEO-based plasmid (Linskens et al. 1999) encoding human soluble FasL (AA 141-281) fused to a hCG-β signal peptide, was used in this study. Generation of this strain is described elsewhere (Lu et al. 2004). All cultures included 10 μg ml^{-1} geneticin (G418) for better maintenance of plasmids.

Media

The complex axenic HL-5C medium (Reymond et al. 1995) contained in g l^{-1}: glucose 10, yeast extract 5, bactotryptone 2.5, casein peptone 2.5, protease peptone 5, KH_2PO_4 1.2, and Na_2HPO_4 0.35. The final pH was adjusted to 6.5 and the medium was autoclaved at 121°C for 20 min and stored at 4°C. Glucose was autoclaved separately and then added to the other sterile components of the medium.

The completely synthetic SIH medium (Han et al. 2004b) was based on the formula of Franke and Kessin (1977), with only slightly changed amino acid composition. The concentration of some limiting amino acids like aspartic acid, glutamic acid, methionine, phenylalanine and, particularly, lysine, was increased.

Supports for immobilization

Broken pumice (diameter 1.5–2.5 mm, pore volume 0.93 g ml^{-1}, particle density 670 g l^{-1}, specific surface area 27 cm^{-1}) was supplied by Raab (Neuwied, Germany). The ceramic carrier CeramTec (Type: F1/porous PST 5; diameter 1.5–2.5 mm, pore volume 0.25 g ml^{-1}, particle density 1,430 g l^{-1}, specific surface area 20 cm^{-1}) was obtained from CeramTec, Wunsiedel, Germany.

Before use, the supports were cleaned with 10% H_2O_2 by gently agitating at 80°C for 0.5 h. After decanting the liquid, the beads were washed with distilled water and dried at 110°C overnight.

Batch cultivation experiments

Cultivations were performed in modified shake flasks (Beshay et al. 2003a) with a total volume of 500 ml. The support particles—10 g CeramTec or 5 g pumice—were placed in a plastic syringe (inner diameter 2.3 cm, length 10 cm) prior to pretreatment with fresh HL-5C medium. The syringe was part of an external loop driven by a peristaltic pump. *D. discoideum* was at first inoculated into 100 ml HL-5C medium in a conventional shake flask of 500 ml at an initial cell density of 8–9×10^4 ml^{-1} and cultivated at 22°C on a rotary shaker (rotational frequency of 150 min^{-1}; excentricity 25 mm) to a certain cell density prior to transferring the culture into the modified shake flask. By using the peristaltic pump, the medium was allowed to circulate through the syringe containing the support in a closed circuit at a flow rate of 5 ml min^{-1}. The cultivation was continued further at 22°C while shaking the flask as described above. After the cell density reached a maximum stationary level in the carrier and in the medium, spent medium was replaced with fresh medium. This modified shake flask cultivation system was applied because the contact of carrier beads with the bottom of the shake flask seemed to affect cell growth of *D. discoideum* (Beshay et al. 2003a).

Continuous cultivation in a trickle bed bioreactor

The trickle bed reactor system shown in Fig. 1 consisted of a glass column (inside diameter 28.1 mm, length 370 mm) fitted with a stainless steel screen at the bottom and an outer jacket for temperature control. As support matrix, 50 g broken pumice were packed into the glass column

Immobilization was carried out by inoculating 300 ml SIH medium in a 1 l Erlenmeyer shake flask with *D. discoideum* at an initial cell concentration of 1×10^5 ml^{-1}. Cultivation was continued at 22°C and a rotational frequency of 170 min^{-1} until a cell density of 2.3×10^7 ml^{-1} was reached. The culture was then

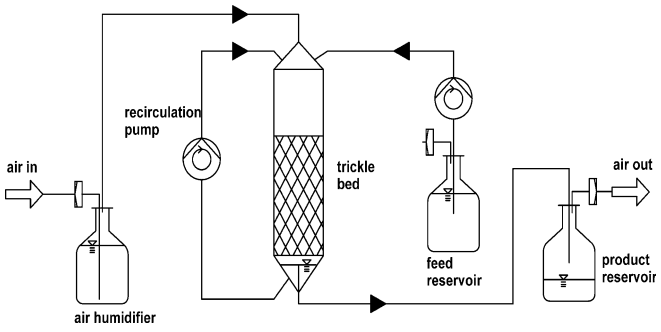

Fig. 1 Flow sheet of the trickle bed reactor system for continuous cultivation of immobilized *Dictyostelium discoideum*

transferred to the glass column shown in Fig. 1 and incubated with the bed of pumice at 22°C for 13 h. During this time, the culture was circulated through the column from top to bottom. At the end of this pre-cultivation, the spent medium was withdrawn and fresh medium was introduced by changing to a continuous mode of operation. Fresh SIH medium and filtered air at a flow rate of 0.8 l min^{-1} were supplied continuously, entering the top of the column. Medium was withdrawn from the bottom of the column from an overflow with a pumping rate higher than the feeding rate in order to arrive at a steady state with a characteristic space velocity fixed by the feed flow rate. The small volume of medium kept in the reactor was circulated by the recirculation pump through the column from top to bottom in concurrent with aeration.

Continuous cultivation in a loop reactor system

A flow sheet of the loop reactor system used to cultivate *D. discoideum* in immobilized state is illustrated in Fig. 2. A glass column (inside diameter 40 mm, length 240 mm) with a water jacket for thermostatting was connected to a laboratory bioreactor of 2 l total volume (proprietary design). A mass of 65 g broken pumice beads corresponding to a bulk volume of 150 ml was packed into the glass column. *D. discoideum* was first allowed to grow in 1 l SIH medium in the 2 l bioreactor only until a cell density of 3–4×10^6 ml^{-1} was observed. The culture medium was then circulated through the column from bottom to top by using a peristaltic pump with a flow rate of 0.35 l min^{-1}. The column was operated as a fixed bed. In the meantime, fresh SIH medium was continuously fed to the bioreactor. The medium volume in the bioreactor was kept constant at 700 ml by running an outlet pump at a higher rate than the feeding pump and fixing a suction pipe at the level to be kept. The space velocity (dilution rate) was changed by changing the feed flow rate. The cultivation was carried out at a temperature of 22°C, a stirrer frequency of 200 min^{-1}, and an air flow rate of 1 l min^{-1}. The pH value was kept constant at 6.5 by automatic addition of 10% H_3PO_4 or 2 M NaOH.

Cell counts and assays

Suspension cell density was monitored by counting cells in a Neubauer chamber. Immobilized cell density in the carrier was determined indirectly by measuring the total protein content of the immobilized cells by means of a modified Bradford test according to Zor and Selinger (1996). Glucose was determined enzymatically using INFINITY glucose reagent (Sigma). The concentration of hFasL in the medium was quantified with an enzyme-linked immunosorbent assay (ELISA) method using a soluble hFasL kit purchased from Diaclone Research (Besançon, France) according to the manufacturer's instructions.

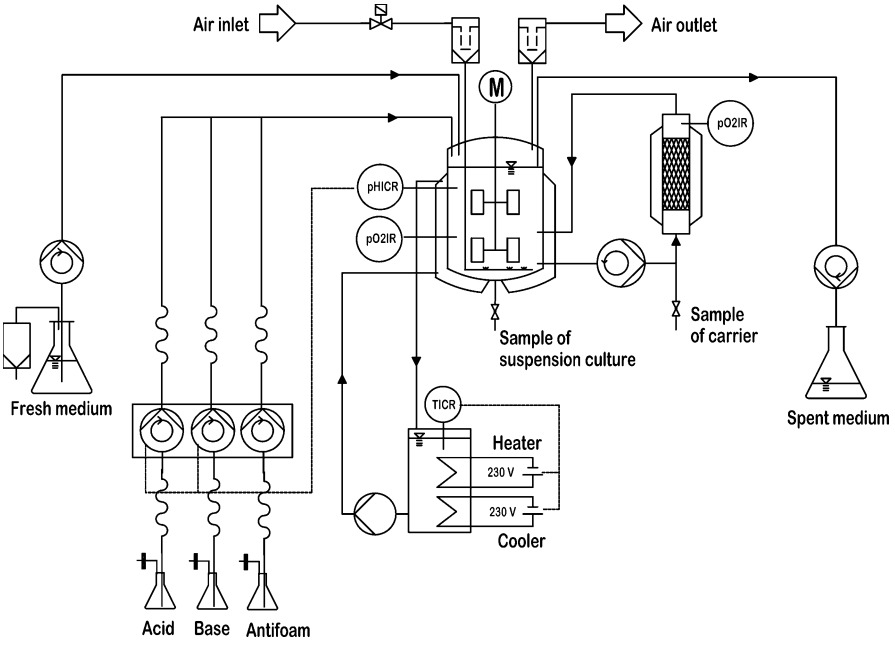

Fig. 2 Experimental set-up of the loop fixed-bed reactor for continuous cultivation of immobilized *D. discoideum*

Results

Batch cultivation of immobilized cells in modified shake flasks

Figure 3 shows cultivations of the hFasL-producing *D. discoideum* strain AX3-pCESFL95-G2 (in short AX3-95G2) in a batch mode of operation carried out in the modified shake flask system. Cells were grown on HL-5C medium in the presence of either broken pumice or the ceramic catalyst carrier CeramTec. The use of CeramTec or pumice as support led to local cell densities of more than 3×10^8 ml^{-1} or 2×10^8 ml^{-1} with respect to the pore volume (N_I), respectively. These values were up to 15–20 times higher than the maximal cell densities observed on HL-5C medium in suspension culture ($1–2\times10^7$ ml^{-1}). Usually, the cell densities in the medium (N_M) were much lower—in the range of 5×10^6 ml^{-1}(Fig. 3). Human Fas ligand was continuously produced by the immobilized cells. After 165 h of cultivation a hFasL concentration of 65μg l^{-1} (with CeramTec as support) or 70μg l^{-1} (with pumice as carrier) was obtained. The production rate of Fas ligand amounted to 20–25 μg h^{-1} l^{-1} pore volume of CeramTec or 15–20 μg h^{-1} l^{-1} pore volume of pumice. The maximum hFasL concentration and the productivity were reproducible after medium exchange. In every repeated batch operation less than half of the initial glucose was consumed.

Continuous cultivation of immobilized cells in a trickle bed reactor

The cultivation of immobilized *D. discoideum* with a minimum of medium volume for the continuous production of hFasL was accomplished in the trickle bed reactor with broken pumice as carrier and with SIH medium (Fig. 4). After incubation for 13 h, an immobilized cell density of 4.7×10^7 ml^{-1} pore volume was measured. At a feed flow rate of 0.018 l h^{-1}, the cell concentration on the carrier increased gradually to 2.2×10^8 ml^{-1} pore volume (N_I) and remained more or less constant when the feed flow rate was changed to 0.031 l h^{-1} and 0.042 l h^{-1}. Thus, immobilized cell densities as high as those in the batch experiments on HL-5C medium were reached.

The effluent human Fas ligand concentration started at less than 40 μg l^{-1} and decreased with increasing feed rate. The hFasL productivity was estimated at 16, 17, and 18 μg h^{-1} l^{-1} pore volume and the glucose consumption rate at 0.83, 0.86 and 0.9 g h^{-1} l^{-1} pore volume. Since the external operating conditions did not change very much with the space velocity, almost constant productivity was observed. The cell density in the medium leaving the reactor continuously, however, changed drastically on a very low basic level as shown in Fig. 4.

Continuous cultivation of immobilized cells in a fixed bed loop reactor system

The above experiment was also performed with broken pumice as support and with SIH medium (Fig. 5). After changing to continuous mode of operation, the cell density in the support quickly rose to 1.6×10^8 ml^{-1} pore volume and could be maintained more or less constant at different space velocities over a total reactor operating time of 500 h (21 days). The hFasL concentration increased with decreasing space velocity and reached a maximum of 101 μg l^{-1} at 0.009 h^{-1}.

The hFasL productivity and glucose consumption rate (estimated as shown in Fig. 6) remained almost constant over a large range of space velocities at 24–25 μg h^{-1} l^{-1} and 0.9–1.1 g h^{-1} l^{-1} pore volume, respectively, but seemed to decrease at low space velocity. Tiltscher and

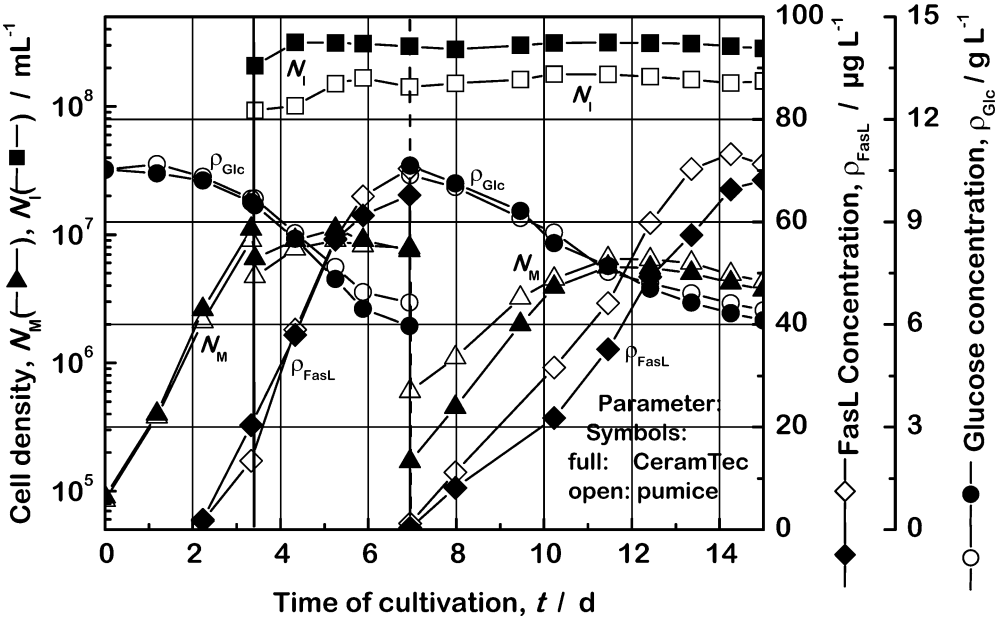

Fig. 3 Repeated batch cultivation of immobilized *D. discoideum* AX3-95G2 on HL-5C medium in the modified shake-flask system with broken pumice and CeramTec as support. *Solid vertical line* Start of external loop pump operation, *broken vertical line* spent medium replaced by the same volume of fresh medium

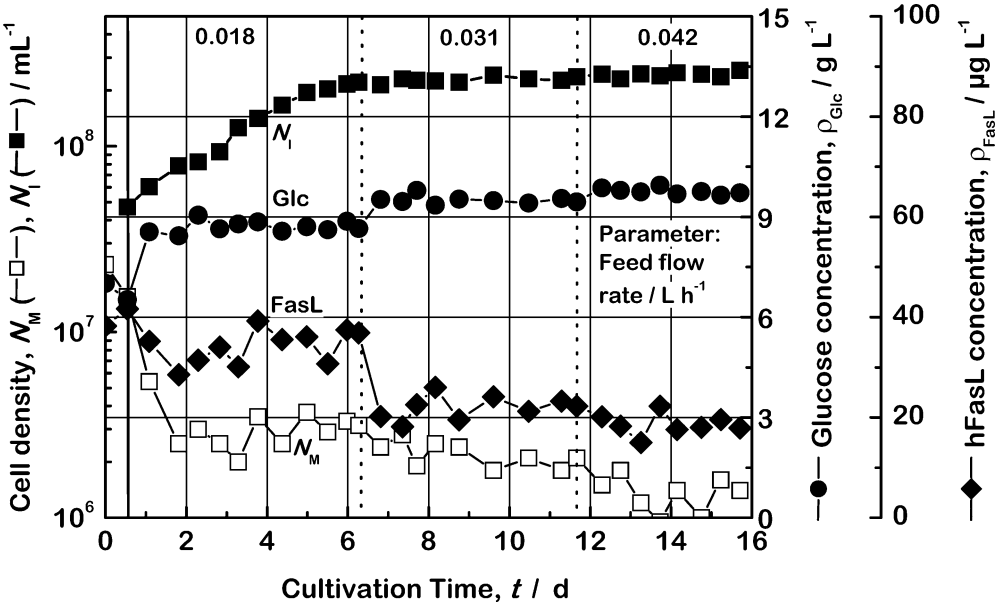

Fig. 4 Cultivation of immobilized *D. discoideum* on SIH medium with pumice as support in a trickle bed reactor with a minimum of outer medium volume at different feeding rates for continuous production of human Fas ligand (hFasL). *Solid vertical line* Culture began circulating through trickle bed, *broken vertical lines* feed flow rate of medium altered

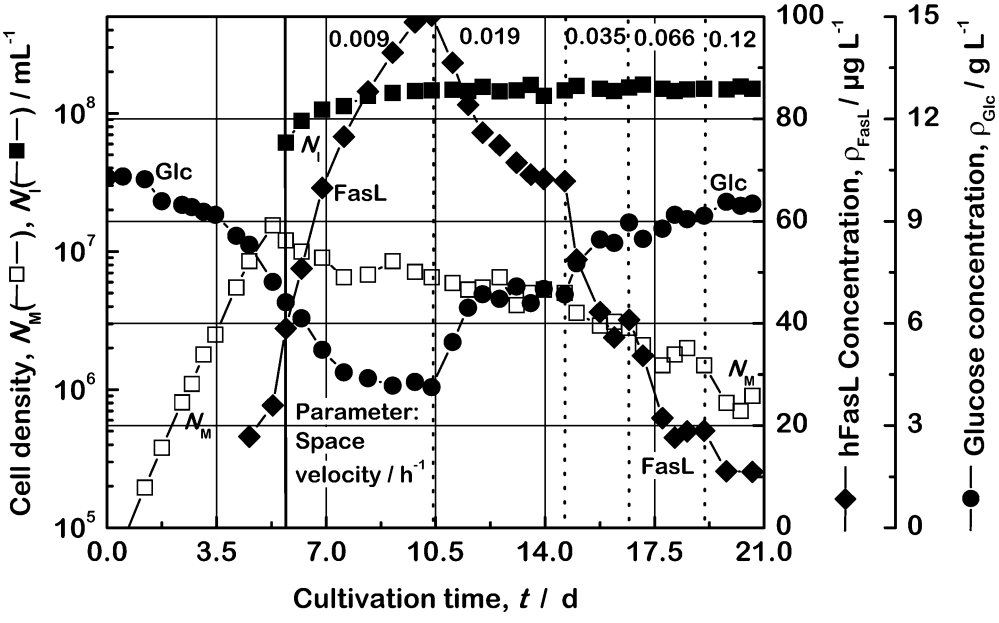

Fig. 5 Continuous cultivation of immobilized *D. discoideum* AX3-95G2 in a loop fixed-bed reactor on SIH medium with pumice as support for the production of human Fas ligand. The *Solid vertical line* Culture began to be circulated through the fixed bed, *broken vertical lines* space velocity of medium altered

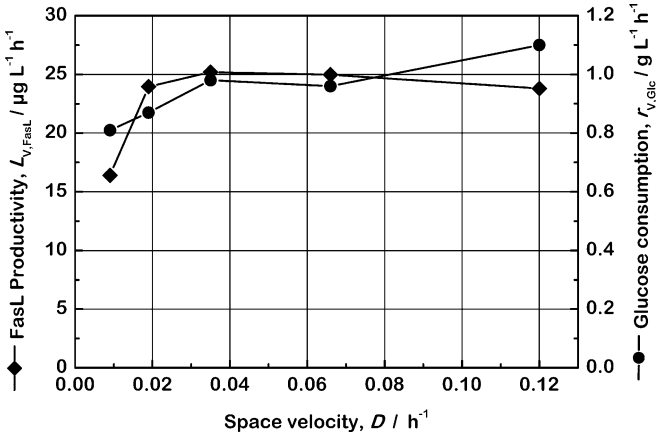

Fig. 6 Profiles of the hFasL productivity and specific glucose consumption rate as a function of space velocity for the cultivation in the fixed bed loop reactor as shown in Fig. 5

Storr (1993) assumed that waste products might accumulate to inhibitory levels at low space velocity, causing a decrease in productivity as well as in glucose consumption. The curves shown in Fig. 6 seemed to represent quite normal profiles that would be expected to originate from zero. The long constant plateau, however, was due to the effect of cell retention by immobilization. The space velocity already exceeded the maximal growth rate, which is obtained in suspension culture of about 0.07 h^{-1}. Thus, wash-out was avoided owing to the presence of immobilized cells.

Discussion

We demonstrate here that broken pumice and the ceramic catalyst carrier CeramTec are supports well suited to the

immobilization of *D. discoideum*. The soluble form of hFasL was produced in repeated batch as well as in continuous mode of bioreactor operation. Either of the following axenic media may be used: complex HL-5C or synthetic SIH. For production of pharmaceutical proteins like hFasL, SIH medium has many advantages over complex media. The immobilized cells reached very high cell densities of more than 3×10^8 ml^{-1} relative to the pore volume in the case of CeramTec, and about 2×10^8 ml^{-1} for pumice. Although the hFasL concentration in the medium was lower compared with that obtained in suspension cultures (Lu et al. 2004), the target protein could be harvested repeatedly or continuously and a constant hFasL productivity of about 20 μg h^{-1} l^{-1} pore volume was observed. The immobilized cell densities and the productivity could be kept constant over a long period of time by repeated medium renewal or continuous operation.

Although SIH medium led to much higher cell densities than HL-5C medium in suspension cultures (Han et al. 2004a), the present results suggest that the cell densities in porous matrices did not depend significantly on whether SIH or HL-5C was used as the cultivation medium. Nonetheless, the growth of *D. discoideum* on synthetic media has now been demonstrated with immobilized cultures. In addition, the continuous cultivation of *D. discoideum* in the immobilized state in a trickle bed reactor with minimum liquid void volume has been established for the production of hFasL.

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